



Real-Time cAMP Dynamics in Live Cells Using the Fluorescent cAMP Difference Detector *In Situ*

Isabella Cattani-Cavaliere¹, Jordyn Margolis¹, Camelia Anicolaesei¹, Francisco J. Nuñez¹, Rennolds S Ostrom^{1,*}

¹Department of Biomedical and Pharmaceutical Sciences, Chapman University School of Pharmacy, Irvine, CA, USA

Abstract

cAMP Difference Detector *In Situ* (cADD_{IS}) is a novel biosensor that allows for the continuous measurement of cAMP levels in living cells. The biosensor is created from a circularly permuted fluorescent protein linked to the hinge region of Epac2. This creates a single fluorophore biosensor that displays either increased or decreased fluorescence upon binding of cAMP. The biosensor exists in red and green upward versions, as well as green downward versions, and several red and green versions targeted to subcellular locations. To illustrate the effectiveness of the biosensor, the green downward cADD_{IS}, which decreases in fluorescence upon cAMP binding, was used. Two protocols using this sensor are demonstrated: one utilizing a 96-well plate reading spectrophotometer that is compatible with high-throughput screening and another utilizing single-cell imaging on a fluorescent microscope. On the plate reader, HEK-293 cells cultured in 96-well plates were stimulated with 10 μ M forskolin or 10 nM isoproterenol, which induced rapid and large decreases in fluorescence in the green downward cADD_{IS}. The biosensor was used to measure cAMP levels in individual human airway smooth muscle (HASM) cells monitored under a fluorescent microscope. The green downward biosensor displayed similar responses to populations of cells when stimulated with forskolin or isoproterenol. This single-cell assay allows visualization of the biosensor location at 20x and 40x magnification. Thus, the cADD_{IS} cAMP biosensor is sensitive and flexible, allowing real-time measurement of cAMP in both immortalized and primary cells, and with single cells or populations of cells. These attributes make the biosensor a valuable tool for studying cAMP signaling dynamics in living cells.

SUMMARY:

The protocol presented in this study illustrates the effectiveness of the cAMP Difference Detector *In Situ* in measuring cAMP through two methods. One method utilizes a 96-well plate reading spectrophotometer with HEK-293 cells. The other method demonstrates individual HASM cells under a fluorescent microscope.

*corresponding author: Rennolds S Ostrom (rostrom@chapman.edu).

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INTRODUCTION:

Adenosine 3',5'-cyclic monophosphate, cAMP, plays a central role in cellular communication and the coordination of various physiological processes. cAMP acts as a second messenger, relaying external signals from hormones, neurotransmitters, or other extracellular molecules to initiate a cascade of intracellular events¹. Moreover, cAMP is intricately involved in various signaling pathways, including those associated with G-protein-coupled receptors (GPCRs) and adenylyl cyclases. Understanding the role of cAMP in cellular signaling is fundamental to unraveling the complex mechanisms that underlie normal cellular functions and the development of potential therapies for a wide range of medical conditions².

In the past, various methods have been employed to measure cAMP directly or indirectly. These included radiolabeling of cellular ATP pools followed by column purification, HPLC, radioimmunoassays, and enzyme-linked immunoassays^{1,2}. These legacy assays are limited by the fact that they are end-point measures, requiring a large number of samples to construct time-dependent responses. More recently, Fluorescence Resonance Energy Transfer (FRET) sensors were developed to create assays in living cells, producing real-time, dynamic data and allowing sensors to be targeted in different subcellular locations³. FRET leverages two fluorophores, one fluorescent donor, and one fluorescent acceptor that when in close proximity, the acceptor fluorophore will be excited by the donor fluorescent output. The two fluorophores most used are cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) since these have compatible excitation and emission properties. In addition to CFP and YFP, the utilization of the green fluorescent protein (GFP) and red fluorescent protein (RFP) is commonly used for FRET biosensors. cAMP FRET biosensors operate by having a donor and acceptor on opposite ends of the Epac2 cAMP binding protein. cAMP binding alters the conformation of Epac and increases the distance between donor and acceptor fluorophores^{3,4}. This conformational change is detected by a loss of FRET, that is, the excitation of the acceptor fluorophore by energy transferred from the donor fluorophore drops³. While a seemingly simple process, there are an abundance of limitations and issues with the FRET biosensor for cAMP research⁵. One of which is the selection of fluorescent proteins, for example, GFP, which can dimerize naturally, thus reducing sensitivity⁶. FRET-based cAMP biosensors have been targeted to specific microdomains⁷, but there may be limitations owing to the large size of a construct with two fluorophores⁶. Another significant issue is the low signal-to-noise ratio of FRET signals resulting from the overlap between excitation and emission of the fluorophore, resulting in high sampling frequency and complicating analysis of the results^{4,5}.

Most recently, the novel biosensor (cAMP Difference Detector *In Situ*, cADDis has solved these and other limitations when it comes to studying the regulation of cAMP signaling⁸. One important improvement is the dependence on a single fluorophore. This allows for a rapid and efficient signal with a wider dynamic range and a high signal-to-noise ratio. As a result, there can be more accuracy as there is a less broad scope of wavelengths to comb through⁸. Like FRET probes, the biosensor has been targeted to subcellular locations, allowing research into the compartmentation theory and exploration in lipid rafts and non-rafts, and other subcellular domains⁹. Perhaps most important is the suitability of a single

fluorophore biosensor for high-throughput screening, which has improved sensitivity and reproducibility over FRET-based biosensors. The biosensor is packaged into a BacMam vector for easy transduction of a wide array of cell types and precise control over protein expression.

Expression control *via* the BacMam vector can be particularly useful in assays using GPCR orthologs from different species to facilitate the interpretation of data from animal studies. Furthermore, control over receptor expression is critical for measuring the different degrees of drug efficacy (e.g., inverse agonists and partial agonists), and low levels of receptor expression are useful to mimic the low levels found in animal tissue. BacMam is a baculovirus vector that has been modified to transduce mammalian cells such as primary cell cultures and HEK-293 lines¹⁰. Dominant selectable markers allow for BacMam to provide more stability over traditional plasmid infections¹¹. Such selective promoters allow for more efficient gene delivery and expression. In addition, adding trichostatin A (a histone deacetylase inhibitor) enhances the reporter protein levels¹¹. Expression levels can be controlled *via* the titer of the Bacmam virus used and should be optimized for each cell type. In the case of cADDIS, a red or green fluorescent protein is linked to the Epac at the N- and C-termini. When cAMP binds, a conformational change in the biosensor moves amino acids adjacent to the fluorescent protein. Such a shift moves the absorbance from the anionic state to the neutral state at 400 nm, thus decreasing the fluorescence.

There are 90–120 GPCRs expressed in a single cell that respond to a wide variety of neurohumoral signals¹². Therefore, it can be hypothesized that at least several dozen GPCRs per cell can stimulate or inhibit cAMP through Gs or Gi coupling, respectively. While there has been progress in monitoring this second messenger in real-time, such as FRET, more efficient methods are needed. The methodology for monitoring the synthesis and degradation of cAMP signals using cADDIS in real-time is presented here. The change in fluorescence can be monitored in real-time using a fluorescence plate reader for high throughput assays or using a fluorescent microscope for single-cell assays. These methods are useful for a wide array of biological questions regarding GPCR signaling *via* cAMP.

PROTOCOL:

The details of all the reagents and equipment used for the study are listed in the **Table of Materials**.

1. Plate reading spectrophotometer high-throughput assay

- 1.1. Seeding HEK-293 cells with the cADDIS BacMam vector in a 96-well plate (Day 1)
 - 1.1.1. Split and infect cells in a viral hood.
 - 1.1.1.1. Warm HEK media (Table 1) and 0.25% trypsin-EDTA in a 37 °C water bath.
 - 1.1.1.2. Disinfect the hood and the materials by wiping them with EtOH-soaked tissues.

- 1.1.1.3.** Remove and discard HEK media from the flask with a serological pipette.
- 1.1.1.4.** Add 5 mL of Mg^{2+} and Ca^{2+} free DPBS (37 °C) with a serological pipette. Tilt the flask back and forth to wash the bottom with DPBS. Remove and discard DPBS with a serological pipette.
- 1.1.1.5.** Trypsinize the cells by adding 3 mL of warmed 0.25% trypsin-EDTA with a serological pipette. Tilt the flask to ensure the reagent fully coats the bottom of the flask. Put the lid on the flask and let the reagent sit for 3–5 min to allow the cells to detach from the 75 cm² flask.

NOTE: This experiment is optimized for a 75 cm² flask; volume adjustments for reagents may need to be made if a different-size culture apparatus is used.
- 1.1.1.6.** Draw 5 mL of fresh media containing antibiotics. Expel and draw the volume of the flask to wash the walls of the flask and physically detach cells.
- 1.1.1.7.** Collect the total volume of the flask (8 mL), and centrifuge (25 °C) in a 15 mL tube at $200 \times g$ for 5 min.
- 1.1.1.8.** After centrifuging, remove the supernatant with a serological pipette, careful not to disturb the pellet.
- 1.1.1.9.** To wash the cells without disturbing the pellet, add 500 μ L of DPBS without Mg^{2+} and Ca^{2+} DPBS to the side of the 15 mL tube. After gently adding the DPBS, remove and discard as much buffer as possible without disturbing the pellet. Repeat three more times.
- 1.1.1.10.** Count the cells and adjust to a cell density of 2,50,000 cells/mL cells.
- 1.1.1.11.** Make a master mix based on what reagent volumes are needed per well of a black, transparent bottom 96-well plate. This is referred to as the “tissue plate.” See volumes below:

NOTE: Example of 1 well (total volume 200 μ L): 40 μ L of cADDIS BacMam vector (stock of 2×10^{10} viral genes /mL), 2 μ L trichostatin A (stock of 100 μ M; final concentration 1 μ M), 158 μ L of 2,50,000 cells/mL cell density. Example of 10 wells (total volume 2 mL): 400 μ L of cADDIS BacMam

vector (stock of 2×10^{10} viral genes /mL), 20 μ L of trichostatin A (stock of 100 μ M), 1,580 μ L media with 2,50,000 cells/mL cell density.

- 1.1.1.12. Add 200 μ L of master mix per well. Incubate the cells at 37 °C and 5% CO₂ in an incubator for 24 h before running on the plate reading spectrophotometer.

1.2. Running on a plate reader

- 1.2.1. On a benchtop, carefully remove all media in each well and replace with 180 μ L of DPBS with Mg²⁺ and Ca²⁺ at 37 °C.
- 1.2.2. Inspect cells on a microscope to confirm cell health.
- 1.2.3. Wrap the plate in aluminum foil and incubate at 37 °C for 30 min–1 h.
- 1.2.4. While incubating, prepare the drugs for the reading at 1:10 dilution (also called 10-fold) for the desired final dose. Thus, prepare 100 μ M forskolin and 100 nM isoproterenol.
- 1.2.5. Add 60 μ L of each drug in a columnar fashion to a clear 96-well plate (referred to as the “drug plate”).

NOTE: With the proper drug dilution and to optimize the reading, the drugs are added to a 96-well plant, referred to as the “drug plate”, to be transferred at the time of the reading to the “tissue plate” using a 96-multi pipettor, allowing the drug to be added to all wells simultaneously and be rapidly read by the fluorescent spectrophotometer.

1.3. Fluorescence plate reader setup

- 1.3.1. Adjust the settings of the fluorescence plate reader.
 - 1.3.1.1. Read Mode: **fluorescence**. Read Type: **kinetic**. Wavelengths reading: **494 nm** and **522 nm**.
 - 1.3.1.2. Reading time: Enter 3 min for the baseline fluorescent read and 7 min for the post-drug addition. “Interval” is entered as **00:00:30** for 30 s between each read.

NOTE: The reading time can be optimized and increased based on the required time.

1.4. Data acquisition

- 1.4.1. Remove the tissue plate from the incubator, remove the aluminum foil and the plate lid, and place the tissue plate in the plate reader.

Close the reader drawer to allow the tissue plate to rest and equilibrate to the reader's temperature (37 °C).

- 1.4.2.** Place the clear 96-well plate (drug plate) with the drug dilutions under the 96-multi pipette and practice drawing 20 μ L from the wells. Ensure that there is an even volume of drug pulled in the pipettor tips.

- 1.4.3.** Initiate a “baseline” measurement run.

NOTE: 40 RFU or above is considered viable data. If data is below this value, the experiment should be stopped and discarded.

- 1.4.4.** The tissue plate will eject from the reader at the end of the baseline run. Swiftly and carefully add 20 μ L from the clear drug plate to the black tissue plate. Then, quickly begin the “treatment” run. The tissue plate should not be out of the reader for more than 30 s after drugs are added.

NOTE: If the drug is added too quickly, the cells detach from the tissue plate, creating an unusable experiment. In addition, the cells are infected with a light-sensitive green fluorescent protein; thus, swiftly and promptly adding the drugs and beginning the second read is imperative to avoid missing the early part of the response.

- 1.4.5.** Once the second reading is complete, ensure that the tissue plate is ejected from the reader and data collection is concluded.

NOTE: Before disposing of the tissue plate, it is best practice to verify that the cells are still attached to the plate. Observe the cells under a microscope. If the cells detach, the results are invalid and should be discarded. The experiment will need to be repeated.

- 1.4.6.** Export the resulting data as an Excel file.

- 1.4.7.** Open the exported Excel readable data from the plate reader and copy and paste relevant information into the Excel conversion template. The data will transform from the plate reader set up to single well, column readouts to the right of the copied data.

NOTE: Columns of fluorescence for a well over time are listed at RFUs (titled column transformation) and as a decimal change relative to the RFU reading at initial reading (t_0 titled Delta F). In the Excel template, the Delta F table is set to the last baseline reading before drug addition. This is the 6th data collection point.

- 1.4.8.** After data transformation, copy the data from Delta F and paste it into data analysis software as decay or relative decay in fluorescence over time.

- 1.4.9.** Change the X- title to “time (s)” and the Y-title to “ $\Delta F/F_0$ ” for relative decimal values.

2. Single-cell assay using an inverted fluorescent microscope

2.1. Seeding (HASM) cells with the cADDIS BacMam vector in a 35 mm dish (Day 1)

2.1.1. Split and infect cells in a viral hood.

- 2.1.1.1. Warm HASM media (Table 1), 0.25% trypsin-EDTA in a 37 °C water bath.
- 2.1.1.2. Disinfect the hood and the materials and wipe the hood with EtOH-soaked tissues.
- 2.1.1.3. Remove and discard HASM media from the flask with a serological pipette.
- 2.1.1.4. Add 5 mL of Mg^{2+} and Ca^{2+} free DPBS (37 °C) with a new serological pipette and tilt the flask to wash the bottom with the DPBS.
- 2.1.1.5. Trypsinize cells by adding 3 mL of warmed 0.25% trypsin-EDTA with a serological pipette. Tilt the flask to ensure the reagent fully coats the bottom of the flask. Let the reagent sit for 3–5 min to allow the cells to detach from the 75 cm² flask.
- 2.1.1.6. Draw 5 mL of fresh media containing antibiotics and expel the pipette to remove the cells from the bottom of the flask.
- 2.1.1.7. Collect the total volume of the flask (8 mL) centrifuge in a 15 mL centrifuge tube at $200 \times g$ for 5 min.
- 2.1.1.8. After centrifuging, remove the supernatant with a serological pipette, careful not to disturb the pellet.
- 2.1.1.9. Without disturbing the pellet, add 500 μ L of DPBS without Mg^{2+} and Ca^{2+} DPBS to the side of the centrifuge tube. Remove and discard as much liquid as possible and repeat one more time.

NOTE: This experiment is optimized for a 75 cm² flask; volume adjustments for reagents may need to be made if a different-size culture apparatus is used.
- 2.1.1.10. Count cells and adjust to 40,000 cells/mL cell density.
- 2.1.1.11. Make a master mix based on the reagent volume needed per well of a 35 mm dish. See volumes below:

NOTE: Example of 1 well (total volume 500 μ L): 20 μ L cADDIS BacMam vector (stock of 2×10^{10} viral

genes /mL), 5 μ L trichostatin A (stock of 100 μ M, final concentration 1 μ M), 500 μ L of 40,000 cells/mL cell density. Example for 4 wells (total volume 2 mL): 80 μ L of cADDis BacMam vector (stock of 2×10^{10} viral genes /mL), 20 μ L of trichostatin A (stock of 100 μ M, final concentration 1 μ M), 2000 μ L media 40,000 cells/mL cell density.

- 2.1.1.12. Add 500 μ L of master mix per well. Incubate the cells at 37 °C and 5% CO₂ for 24 h before continuing the experiment.

NOTE: The cell number may vary according to the cell line. To avoid overlapping cells for the analysis, use low cell numbers.

2.2. Preparing pharmacological agents (Day 2)

- 2.2.1. Carefully remove media in each well and replace 450 μ L of DPBS with Mg²⁺ and Ca²⁺ at 37 °C.
- 2.2.2. Wrap the plate in aluminum foil and incubate the 35 mm dish at 37 °C for 30 min–1 h.
- 2.2.3. Inspect cells on a microscope to confirm cell health.
- 2.2.4. In the meantime, prepare the drugs for the reading 1 log unit above the desired final concentration (10-fold greater). Thus, prepare 100 μ M forskolin and 100 nM isoproterenol.
- 2.2.5. For log dose titers, prepare the drug at 1 mM and use serial dilutions to achieve 100 μ M of forskolin and 100 nM of isoproterenol.

2.3. Data acquisition

NOTE: The following settings are for a fluorescent inverted microscope. The software accompanying each microscope can differ; the general settings used in the current protocol are described here.

- 2.3.1. Turn on the central hub, then the inverted fluorescent microscope.
- 2.3.2. Open the software that will be used for the capture and choose the **time-lapse capture** option.
- 2.3.3. Place a 35 mm dish sample holder in the microscope stage.
- 2.3.4. Set objective lens to **x20**.
- 2.3.5. Use **autofocus** and get as clear a picture as possible. If it is difficult to find individual cells, start on a 4x objective, then move to 20x. Focus the sample manually if the microscope lacks an autofocus feature.

NOTE: The magnification of 40x can be used if more significant cell morphological detail is needed.

2.3.6. Adjust the following settings for obtaining optimal data acquisition: **Auto Brightness** (exposure), **Excitation Wavelength**, **Black Balance** (to reduce background noise), **Auto Focus**.

2.3.7. After finding an area of view with several cytoplasmic fluorescing cells, acquire data using time-lapse capture for 20 min every 30 s.

2.3.8. Click on **go** to begin the reading.

2.3.9. Run for 3 min to collect baseline. As soon as the 3 min capture is taken, gently add 50 μ L of the drug to the appropriate well. When the drugs are added, the final concentration on the dish will be of 10 μ M forskolin and 10 nM of isoproterenol.

NOTE: The drug must be added carefully; do not touch the plate with the pipette tip, as this may move the field of view made and make the sample unable to be analyzed.

2.3.10. Leave the experiment to run for 17 more minutes with data capture every 30 s.

2.3.11. Once the test is completed, ensure that the data is ready for analysis.

NOTE: While the current protocol does not incorporate a control for focal drift, it could be beneficial to include one in the experiment. For instance, the use of nuclear dyes can assist in managing focal drift during microscope analysis, especially when capturing images of live cells over prolonged durations. cADDIs biosensors are typically distributed widely within the cell, making a broad focal plane the most effective capture and reducing the need for controlling focal drift⁹.

2.4. Analyzing the data

NOTE: The software accompanying each microscope can differ, so the general settings that should be used to analyze the captured images are described here.

2.4.1. Open the image files captured during the experiment.

2.4.2. Use the **polygon** or **circle** tool to select the region of interest of each cell to perform the analysis. The analysis should give the brightness of each cell at each time point.

NOTE: Best practice is to select individual cells and not cells touching the wall of the dish or other cells. This ensures the best data collection possible. Select a minimum of 5 cells for analysis within each condition, and replicate the experiment at least three times.

- 2.4.4. After analyzing the data, open the data in an Excel sheet and calculate the average brightness for each condition at each photo, starting at the picture just before adding the pharmacological agents or vehicle. In this example, at the 3 min mark or the 6th photo taken on the microscope, since each picture is taken within 30 s.
- 2.4.5. Calculate $\Delta F/F_0$ for each average value in each condition.
- 2.4.6. Load into a statistical analysis software and plot $\Delta F/F_0$ against time in s.

NOTE: Figure 1 provides a schematic overview of the major steps of the protocols.

REPRESENTATIVE RESULTS:

The present study validated the cytosolic cADDis biosensor in both plate reader and microscope assays. Once cells expressed the biosensor, they were stimulated with either 10 μ M forskolin (a direct activator of adenylyl cyclase), 10 nM isoproterenol (an agonist at β_1 AR and β_2 AR), or vehicle (Figure 1). The subsequent changes in fluorescence, indicative of cAMP production, were captured every 30 s.

The data was transformed as the change in fluorescence from the initial fluorescence ($\Delta F/F_0$). A one-site decay model was applied to the fluorescence data to quantify the temporal changes in cAMP levels across the different conditions (Figure 2 and Figure 3). The parameters of these decay curves can be used to quantify the response to a given concentration of the drug. The product of the decay rate (k) and the plateau as a single value that quantifies the drug response was used, and these were used to create concentration-response curves when multiple concentrations of the same drug are applied¹³.

On the plate reader, upon stimulation with 10 μ M forskolin, the cytosolic sensor showed a sizable decrease in fluorescence intensity within seconds and progressed over many minutes (from the baseline 0 $\Delta F/F_0$ at 200 s to -0.7 $\Delta F/F_0$ at 600 s) in HEK-293 cells (Figure 2A). Given the green sensor's design, a decrease in fluorescence indicates an increase in cAMP production, indicating that forskolin caused a uniform increase in cAMP levels in the cytosol. Stimulating HEK-293 cells with a sub-maximal concentration of isoproterenol (10 nM) led to rapid but smaller decreases in cADDis fluorescence (-0.3 $\Delta F/F_0$ at 600 s, Figure 2A). When these lower concentrations of isoproterenol (10 nM and 100 nM) were examined in a single well of cells over time, oscillations of cAMP levels were observed in HEK-293 cells (Figure 2B). The periodicity of these oscillations was consistently around 200 s. The routine data analysis includes fitting the response to a single site decay model, which averages these oscillations (see connecting line). Averaging multiple experiments together typically led to these oscillations becoming obscured in the data. Nonetheless, the biosensor displays a sensitivity and rapid kinetics that allow the observation of cAMP oscillations.

The biosensor can also be measured using a fluorescence microscope. This approach allows monitoring of cAMP in single cells and can also be adapted to visualize biosensors that are targeted to different subcellular locations. In the present study, the biosensor was

used in primary human airway smooth muscle (HASM) cells. Stimulation of HASM with either vehicle, 10 μ M forskolin, or 10 nM isoproterenol leads to an observable decrease in fluorescence intensity over time (from the baseline 0 $\Delta F/F_o$ at 120 s to $-0.9 \Delta F/F_o$ at 410 s) (Figure 3) (**Video 1**, **Video 2**, and **Video 3**). Fluorescence is evenly distributed throughout the cytosol of cells and excludes the nucleus (see the time-lapse videos). Thus, forskolin and isoproterenol produced a similar rapid and sizable decrease in fluorescence HASM cells. These data demonstrate the ability to use cADDIs in primary cell cultures.

DISCUSSION:

Accurate and sensitive measurement of cAMP is crucial for understanding its role in various cellular processes and for studying the activity of cAMP-dependent signaling pathways. There are several methods commonly employed to measure cAMP levels, including ELISA, radioimmunoassay, FRET biosensors, and the GloSensor cAMP assay^{14–18}. Each cAMP assay has strengths and weaknesses. The protocol allows the real-time detection and monitoring, ranging from minutes to hours, and measurement of cAMP dynamics within living cells without the need to include phosphodiesterase inhibitors. This latter point is critical for researchers interested in studying the role of phosphodiesterase isoforms in regulating cAMP signaling¹⁹. The biosensor is available commercially (see **Table of Materials**) in multiple versions: a green fluorophore that decreases fluorescence upon cAMP binding, a red fluorophore that exhibits increased fluorescence upon cAMP binding, and a green fluorophore that exhibits increased fluorescence upon cAMP binding. The downward version of the biosensor is most useful since the main limiting factor is the level of expression of the biosensor, and this version will display maximal fluorescence at baseline prior to starting an experiment, alleviating the need to assess biosensor expression by adding a maximal cAMP stimulus. The biosensor is also available in versions that are targeted to membrane microdomains, primary cilia, or to the nucleus. These different versions enable measurements of cAMP simultaneously in cytosolic and membrane/cilial compartments within the same cell. These novel tools are uniquely adept at the visualization and quantification of cAMP dynamics across different cellular components, a rapidly evolving and important area of study²⁰.

The biosensors offer a straightforward method to effectively monitor real-time cAMP dynamics, enabling researchers to observe rapid changes in cAMP levels with exceptional temporal resolution. One of the key strengths of the biosensor lies in its high sensitivity, which allows for the detection of both subtle and significant variations in cAMP concentrations⁸. An example of this in the cAMP oscillations is observed at low levels of isoproterenol stimulus in HEK-293 cells with no phosphodiesterase inhibitor present (Figure 2B). This oscillatory behavior is not understood and appears unique to these cells, but the sensitivity and rapid kinetics of the biosensor enable their detection. It should be noted, however, that the biosensor can be readily saturated, so this biosensor can be limited in quantifying the differences between medium and large cAMP signals. This limiting feature is particularly relevant when employing phosphodiesterase inhibitors that cause global increases in cAMP levels when a receptor stimulus is also present. While we present the infection of HASM in this study, it's important to acknowledge the challenge associated with infecting primary cells using the BacMam vector. The Conditions should

be optimized for individual cell types in order to achieve sufficient expression of the biosensor. Nonetheless, the sensitivity and scalability of the biosensor make it well-suited for high-throughput assays.

Another improvement of using this biosensor over FRET-based sensors is the minimization of photobleaching. Many fluorescent biosensors are subject to photobleaching following prolonged and repeated excitation. The protocols described here involve short-duration excitations that are spaced 30 s apart. The biosensor is also extremely bright, thus limiting potential photobleaching for this type of analysis. Photobleaching would be observed during the initial baseline read as a drift. If baseline drift were to occur, one could subtract the drifting baseline from the data before fitting the curve or increase the sampling interval to reduce the frequency of excitation.

Like other cAMP biosensors, this biosensor has high specificity for binding to cAMP. Consequently, the signals generated by this assay directly correlate to cAMP levels, significantly reducing the potential interference from other cellular components and providing reliable results. Furthermore, the versatility of the biosensor is worth highlighting. While the GloSensor assay is widely used in the field, it does have certain limitations. For instance, relying on transient transfection may result in inefficiencies in specific cell types, and the analysis of multiple parameters or processes within the same cell is a challenge. Additionally, the assay relies on luciferase enzymes that can be influenced by certain compounds, potentially leading to compromised result accuracy²¹. On the other hand, the biosensor, can be employed in various cell types and experimental systems, including adherent and suspension cells. Its compatibility with diverse cellular contexts allows researchers to investigate cAMP dynamics across different biological systems^{22–24}. The integration of the cADDIS assay with fluorescence microscopy enhances its utility even further since cell morphological information can also be gathered^{25–27}.

The cADDIS assay provides researchers with great flexibility in experimental design, enabling a wide range of investigations related to cAMP signaling. It serves multiple purposes, allowing for the measurement of basal cAMP levels, the study of cAMP fluctuations in response to diverse stimuli or treatments, and the exploration of cAMP dynamics in various physiological or pathological conditions. One of the key strengths of the assay is its compatibility with other techniques, which allows for a more comprehensive understanding of cAMP signaling. By combining the biosensor with methods such as immunofluorescence staining or genetic manipulation¹⁰, researchers can gain deeper insights into the precise spatial and temporal regulation of cAMP within specific cellular compartments or signaling pathways. Through this integration, researchers can investigate the diverse functions of cAMP in various contexts, leading to valuable insights into the complexities of cAMP-mediated cellular processes. Employing these techniques allows researchers to visualize and quantify cAMP levels at both the individual cell and population level, offering a comprehensive understanding of its distribution within the cell, including the identification of cAMP pools. As a result, the combination of these methods enables the acquisition of precise data, contributing significantly to our knowledge of cAMP signaling.

While traditional methods have been valuable for cAMP analysis, the introduction of cADDIS represents a promising advancement in this field. Its specificity, sensitivity, and real-time capabilities make it a valuable addition to the researcher's toolkit, particularly due to its compatibility with living cells. With the ability to monitor cAMP levels in real-time and its high sensitivity, the biosensor offers a significant advantage in various experimental settings. This progress contributes to a deeper understanding of the dynamic nature of cAMP signaling and its functional implications in both physiological and pathological processes.

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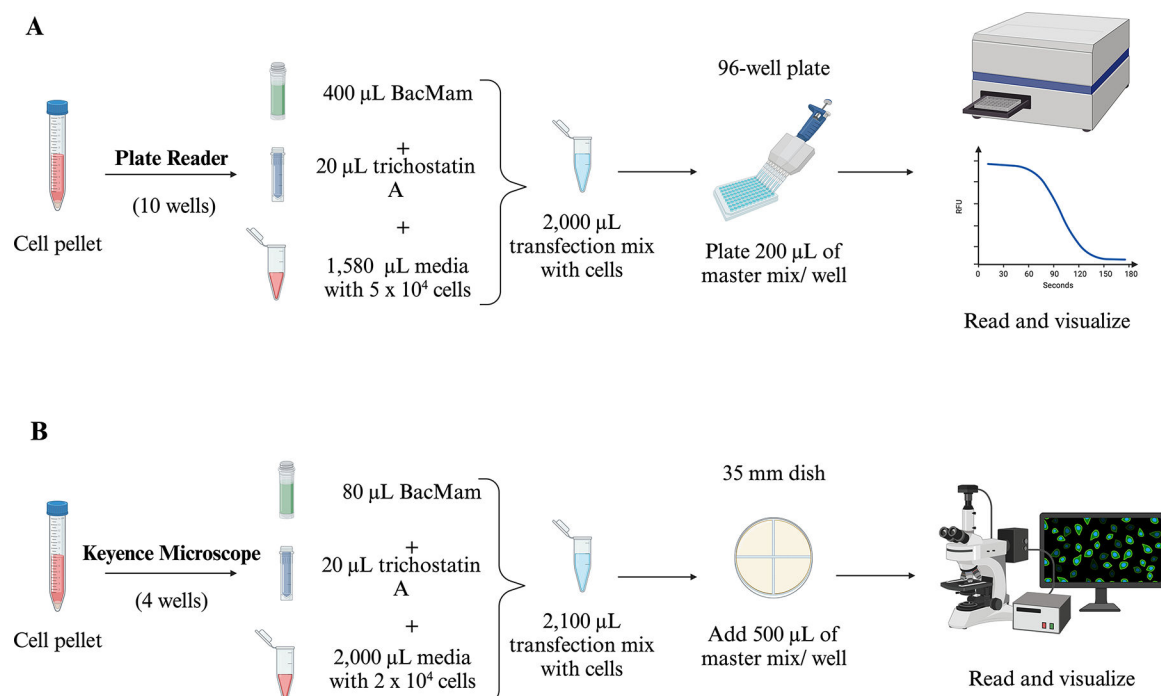


Figure 1: Schematic representation of the biosensor protocol steps.

Initially, culture cells are split and resuspended with fresh media, then infected with BacMam virus carrying the cADDIs sensors. Post-transfection cells are stimulated with pharmacological agents triggering cellular pathways that alter cAMP. Changes in the fluorescence intensity in response to the varying cAMP concentration are captured using a plate reading spectrophotometer (**A**) or fluorescence microscope (**B**), providing a real-time quantification of the cAMP dynamics.

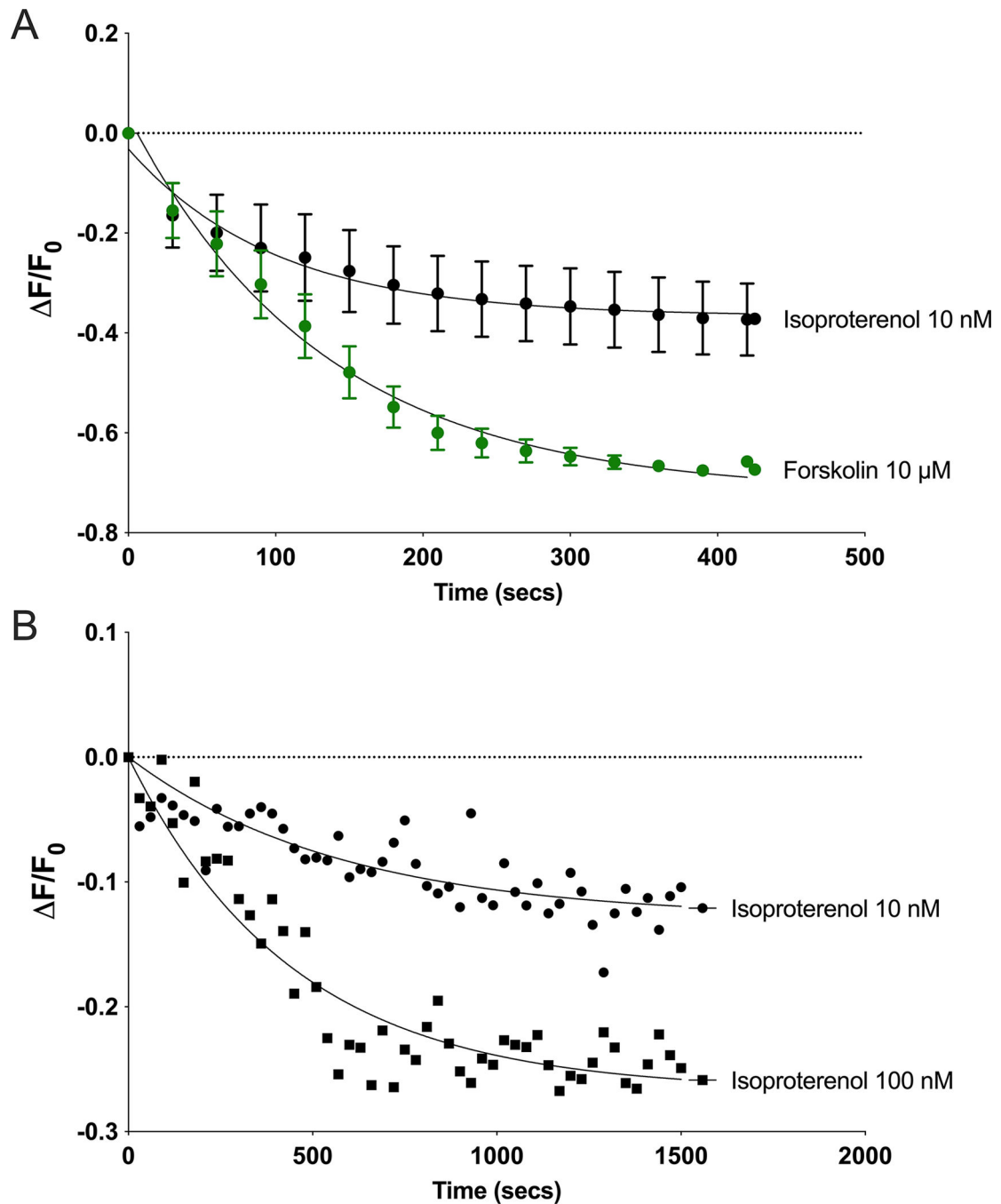


Figure 2: Real-time monitoring of cAMP in live HEK-293 cells on a plate reading spectrophotometer.

Fluorescence of HEK-293 cells on 96-well plates expressing the biosensor was measured over time. After baseline fluorescence was established, fluorescence decay was monitored for 7 min. **(A)** Fluorescence decay after the addition of either 10 μ M forskolin or 10 nM of isoproterenol is plotted as the mean $\pm$ SEM of 10–15 experiments. **(B)** Fluorescence decay oscillations are shown by plotting a single experiment after the addition of either 10 nM or

100 nM of isoproterenol. Data in both graphs is fit to a single site decay model (connecting line).

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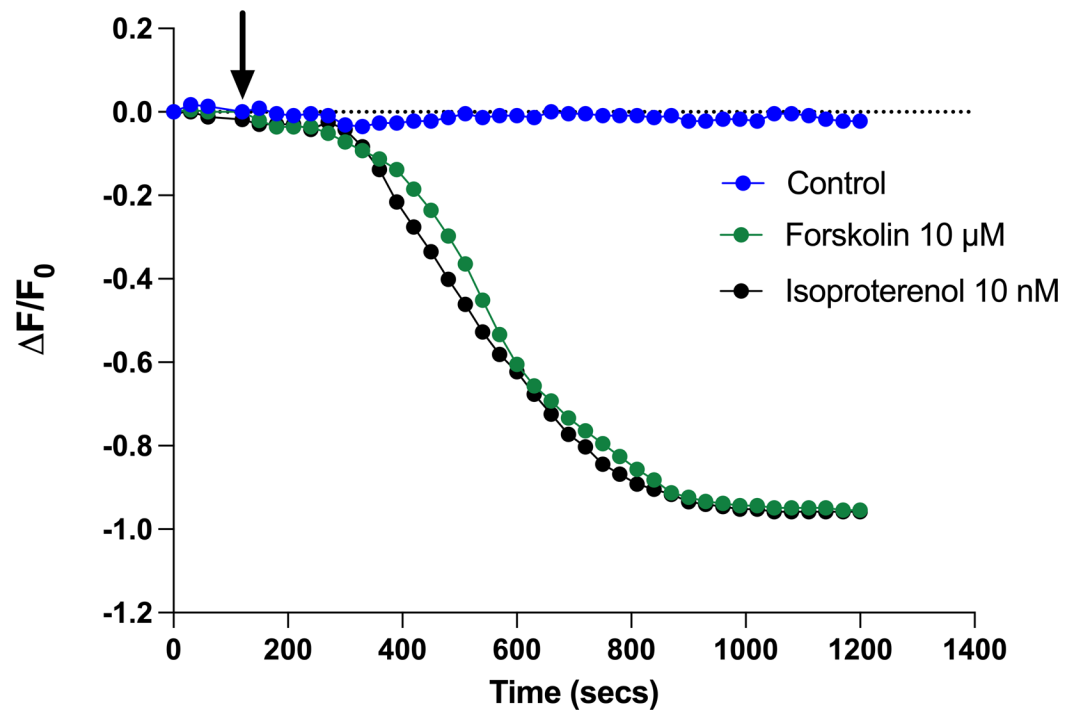


Figure 3: Real-time monitoring of cAMP in live HASM cells on a fluorescence microscope. Fluorescence of HASM cells on plated 35 mm dishes expressing the biosensor was measured over time. After baseline values were established, fluorescence decay was measured every 30 s for 20 min. The indicated agent was added at 120 s (arrow). The biosensor fluorescence decay curves in response to either vehicle, 10 μM forskolin, or 10 nM isoproterenol. Data from a single experiment is shown with a connecting line. Time-lapse videos of cells treated with vehicle (control), forskolin, or isoproterenol are included to provide a visual representation of the fluorescent responses of the biosensor.

Table 1:
Media formulation of HASM medium and HEK medium.

Media formulation	
HASM medium	
Name	Volume
Ham’s F-12K	419.65 mL
FBS	50 mL
HEPES (1M)	12.5 mL
Sodium hydroxide solution	6 mL
L-glutamine 200 mM (100X)	5 mL
Calcium chloride (1 M)	0.850 mL
Antibiotic-Antimycotic (100X)	5 mL
Primocin	1 mL
HEK medium	
Name	Volume
DMEM (1x)	444 mL
FBS	50 mL
Antibiotic-Antimycotic (100X)	5 mL
Primocin	1 mL